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OVOMUCIN, A SUBSTRATE FOR THE ENZYME OF INFLUENZA VIRUS. I. OVOMUCIN AS AN INHIBITOR OF HAEMAGGLU- TINATION BY HEATED LEE VIRUS.

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It was previously shown by Hirst (1942) (1) that influenza viruses are adsorbed to chicken red blood cells and elute spontaneously after a period of time, the process of elution having a temperature coefficient of about 3 over the range 27–37° C; (2) that the virus after its spontaneous elution from the cell surface is functionally intact, whereas the red cell is irreversibly changed so as to be no longer available for virus adsorption. In interpreting his exacting observations Hirst compared the interaction between the virus agglutinin and the receptor substance at the surface of the red cells with an enzyme-substrate interaction, the enzyme possessed by the virus changing the properties of the cell receptor substance.

In a series of investigations Burnet and co-workers (Burnet, McCrea and Stone, 1946; Stone, 1947; Anderson, 1948; Burnet, 1948a; Burnet, 1948b) have advanced much evidence to substantiate Hirst's suggestion of the presence at the virus surface of a true catalyst. The method used by Burnet to test for virus enzyme activity is based on the findings (1) that influenza virus type B, when heated at 55° C. for 30 minutes, fully retains its haemagglutinating properties, but loses its ability to elute spontaneously (Briody, 1948); (2) that certain substances are able to inhibit haemagglutination by heated virus, when mixed together with the virus prior to the addition of the red blood cells. The first observation relevant to (2) was made by Francis (1947), who showed the power of normal human serum to inhibit haemagglutination by heated influenza B virus. McCrea (1948) proved the inhibitor present in serum to be a component of the heat-stable mucoprotein fraction of human serum and prepared (McCrea, 1949) from ovarian cyst fluid a purified mucopolysaccharide highly active as "virus haemagglutinin inhibitor," i.e. a substance able to combine with and to fix heated influenza B virus, thereby inhibiting the agglutination of added red blood cells. Burnet (1948a) demonstrated the inhibitory power of purified blood group O substance, a mucopolysaccharide prepared from human ovarian cysts

by Morgan (King and Morgan, 1944 ; Morgan, 1947 ; Aminoff and Morgan, 1948), and de Burgh, Yu, Howe and Bovarnick (1948) provided evidence for the polysaccharide nature of a very efficient inhibitor which can be extracted from human red cells and may be identical with the receptor substance of erythrocytes (Hirst, 1948).

The important fact disclosed by Burnet and co-workers (Anderson, 1948 ; Burnet, 1948*a*, 1948*b*) is the loss by previous treatment with active (unheated) influenza virus of the ability of the various mucoproteins and mucopolysaccharides to act as virus haemagglutinin inhibitor. Whatever may be the forces of attraction between the surface of the heat-inactivated virus and the mucoid molecule, the lack of association after treatment with active virus scarcely allows of another interpretation than of a structural change in the substrate. This finding, taken together with the further observation that a soluble enzyme of *Vibrio cholerae*, referred to as receptor destroying enzyme or RDE (Burnet and Stone, 1947), closely imitates active influenza virus in its ability to reduce the power of mucoids to inhibit haemagglutination by heated virus (Anderson, 1948 ; Burnet, 1948*a*), strongly suggests the enzymatic nature of the process.

At this stage it seemed desirable to investigate the chemical change involved in the interaction between active influenza virus and suitable mucoproteins. It is the aim of the experimental work to be described in a series of papers to present information regarding the composition of the product of this interaction, thus affording conclusive evidence for the enzymatic character of the virus activity under discussion and obtaining some insight into the chemistry of the underlying reaction. For such an investigation it was essential to provide a virus inhibitor easily available and readily acted upon by the virus concerned. In the search for such a substance use was made of the observation by Burnet (unpublished) that hen egg-white is an efficient virus haemagglutinin inhibitor, a property rapidly lost by pre-treatment with active virus or with RDE. Lanni and Beard (1948) have also reported a similar inhibitory effect of egg-white on haemagglutination by heated swine influenza virus. In this paper it will be shown that of the various egg-white proteins the ovomucin fraction is mainly responsible for these phenomena. Since the preparation of this fraction presents no difficulty, ovomucin fulfils the specified requirements.

MATERIALS.

Preparation of ovomucin.

Ovomucin was prepared following mainly the procedure described by Young (1937). 600 ml. egg-white, obtained from about 20 fresh white Leghorn eggs and freed of the chalazae, were chilled and poured into 1600 ml. distilled water at 0°C. The precipitate was centrifuged off and washed three times with cold 0.5 per cent NaCl solution in the centrifuge. The washed precipitate was dispersed in the minimal volume of 10 per cent NaCl solution and reprecipitated by adding the dispersion to about twenty volumes of distilled water at 0° C. After centrifugation the snow-white precipitate was washed with water until washings were chlorine free and practically devoid of protein. The final product was dried over conc. H₂SO₄ *in vacuo*. This preparation, of a white, silky appearance was analyzed for moisture, ash, nitrogen, hexosamine and reducing substances with the following result :

TABLE I.—*Analysis of the Ovomucin Fraction of Egg-white.*

	Moisture. mg.	Ash. mg.	Nitrogen. mg.	Reducing substance (as glucose). mg.	Hexosa- mine. mg.
100 mg. ovomucin contain	6.3	1.64	13.20	15.4 (15.1*)	11.8

* Hydrolysed with 2N HCl at 100° C. for 5 hours.

The figures in Table I are in fair agreement with the data given by Young (1937). Ovomucin, once dried, even if drying is gently effected from the frozen state, is scarcely soluble in water or in NaCl solutions of various concentration. It was found that the capacity of ovomucin to inhibit haemagglutination by heated virus and the loss of this capacity by pre-treatment with active virus was changed neither quantitatively nor qualitatively, if the precipitates were washed only once. Therefore, for experiments concerned mainly with the kinetics of the virus-substrate interaction the second precipitate, after one washing, was redispersed in an appropriate volume of NaCl solution to give a final ovomucin concentration of 0.5 to 1.0 per cent and a final salt concentration of 5 per cent. This dispersion, showing a considerable viscosity and a faint opalescence, was dialysed in a cellophane tube for 48 hours at 4° C. against two changes of 1.8 and 1.0 per cent NaCl solution respectively.

It must be pointed out, however, that ovomucin, prepared from egg-white by precipitation with water, represents a protein fraction rather than an individual protein. As was shown in this Institute by Mr. E. L. French, all ovomucin preparations contain lysozyme in various concentration, up to 15 per cent of the total ovomucin fraction. The lysozyme content of this fraction may be considerably decreased by shifting the pH of the final dispersion (in 5 per cent NaCl) to 9.7 with 1 N KOH, chilling and seeding the liquid with crystals of isoelectric lysozyme. Under these conditions lysozyme crystallizes out from the dispersion within 2 or 3 days at 4° C. and is removed together with some gelatinous matter by centrifugation. By this method, described for egg-white by Alderton and Fevold (1946,) the lysozyme content of the ovomucin fraction can be reduced to about 4 per cent of the total. The dispersion, after readjustment of the pH to 7.0, is then dialysed, as described above. This procedure yields a rather uniform dispersion of the ovomucin fraction, which may be diluted with 0.85 per cent NaCl solution, if so required.

It may be mentioned that separation or concentration of the virus haemagglutinin inhibitor component of the ovomucin fraction by chloroform extraction (Sevag's (1934) method as modified by Bay, Henry and Stacey (1946)) is not feasible. If an ovomucin dispersion (inhibitory titre 6400) was shaken for 30 minutes with 1/5 of its volume of chloroform and 1/50 of its volume of *n*-butanol and then centrifuged for 5 minutes, only 5 per cent of the inhibitor was recovered in the upper layer (inhibitory titre 300). Submitting the upper layer to the same treatment further reduced the inhibitory power (inhibitory titre <50).

Preparation of ovomucoid, egg albumin and lysozyme.

Ovomucoid was prepared according to Mörner (1894), crystalline egg albumin according to Cole (1933) and crystalline lysozyme (isoelectric) from egg-white according to Alderton and Fevold (1946).

Purification and concentration of influenza virus.

Two strains of influenza virus, Melbourne (type A) and Lee (type B) were used. In each case purification and concentration were carried out in the following manner: 75 ml. of a centrifuged pool of allantoic fluids harvested from 13-day-old chick embryos, inoculated with the respective virus 2 days previously, were mixed with 75 ml. of 0.1 M phosphate buffer (pH = 8.2). 0.1 M calcium chloride solution was then added drop by drop with constant stirring in two portions of 6.8 ml. and 4.5 ml. respectively and the precipitate resulting from each addition spun down in a refrigerated centrifuge. After washing with 0.1 M phosphate buffer (pH = 8.2) the precipitate was quickly dissolved in 10 ml. of half-saturated ammonium citrate solution, previously adjusted to pH 6.3 with citric acid, and the mixture dialysed for 60 minutes against running tap water and for 48 hours at 4° C. against two changes of 4 l. 0.8 per cent NaCl solution. The whole procedure was performed under sterile conditions, and only preparations free from bacterial contamination were used in the experiments. The technique is a modification of Salk's (1941) method, first applied in this Institute by Dr. McCrea.

Preparation of the receptor destroying enzyme (RDE).

A culture filtrate of a suitable strain of *Vibrio cholerae* was heated at 55° C. for 30 minutes in the presence of excess calcium. From this material a red cell eluate was prepared according to Burnet and Stone (1947).

METHODS.

Nitrogen determinations were made by the micro-Kjeldahl method, using the apparatus of Parnas and Wagner. Period of combustion was 8 hours after the addition of the catalyst (selenium dioxide).

The reducing power was determined by the Hagedorn-Jensen micromethod after hydrolysis of the substance with 1.5 N HCl at 100° C. for 3½ hours in a sealed tube.

The hexosamine content of ovomucin was estimated according to Elson and Morgan (1933). Hydrolysis prior to the determination was effected by 0.5 N HCl at 100° C. for 40 hours, conditions found to give maximum value. The figures, both for the reducing power and the hexosamine content, as tabulated, are not corrected for the unavoidable loss of carbohydrate in the course of acid hydrolysis under the conditions prevailing.

Titration of inhibitory activity of ovomucin.

Titration was performed essentially according to the technique described by Burnet (1948a). Serial twofold dilutions of the inhibitor, with 0.85 per cent NaCl as diluent, were prepared in 0.25 ml. volumes. To each tube was added one drop of Lee infected allantoic fluid, previously heated at 56° C. for 30 minutes and diluted so as to contain five agglutinating doses of virus per drop. After shaking, the mixtures were held for 30 minutes at room temperature, when 0.25

ml. of 1 per cent suspension of "sensitive" fowl red cells (Anderson, 1948) was pipetted into each tube. The cells were then allowed to settle for one hour at room temperature, and the end-point, i.e. 50 per cent haemagglutination, was determined by observation of the pattern of the deposited cells. The reciprocal of the inhibitor dilution showing the end-point was recorded as the inhibitory titre of the preparation. The dilutions were referred to that inhibitor concentration actually used in the experiment, unless otherwise stated.

For the determination of the residual inhibitor in the presence of virus the sample was diluted 1 : 10 with 2 per cent NaCl solution (unless stated otherwise) and heated at 65° C. for 30 minutes to destroy the virus. Controls were treated identically. Titrations were then carried out as above. The inhibitor was only slightly affected by this heat-treatment.

Titration of virus haemagglutinin.

Haemagglutinin titrations of virus were performed according to the method of Burnet, Beveridge, Bull and Clark (1942) with the modification that a suspension of 1 per cent fowl red cells was used.

Titration of RDE activity.

The activity of RDE was determined, as described by Burnet and Stone (1947).

EXPERIMENTAL.

The inhibitory power of ovomucin as compared with that of the other egg-white proteins.

TABLE II.—*The Virus Haemagglutinin Inhibitory Titres of the Various Egg-white Proteins.*

Protein	Inhibitory titre referred to 1 gm. of protein (dry weight)
Ovomucin fraction . . .	2.4×10^6 to 4.8×10^6
Ovomucoid . . .	1×10^3
Ovalbumin . . .	$< 2 \times 10^2$
Lysozyme . . .	$< 2 \times 10^2$

It may be seen from Table II that of the various proteins present in egg-white it is the ovomucin fraction which is endowed with the power to inhibit haemagglutination by heated influenza virus. As will be shown in a following paper, this inhibitory power is a property of only a small portion of the ovomucin fraction. Ovomucin, as usually prepared, is not an individual protein, but rather a protein-mucoprotein mixture. The lysozyme content of the ovomucin fraction can be reduced, as described under "materials." Its complete removal was not attempted, since the residual lysozyme, while representing only a minor impurity, most efficiently protects ovomucin against contamination by air-borne bacteria. It is due to the lysozyme content of ovomucin that larger amounts of this fraction could be prepared without taking sterile precautions and that the incubation of the final product with virus at 38° C. for several hours did not require the addition of an antiseptic.

Reduction of the inhibitory power of ovomucin by active virus.

An approximately 1 per cent dispersion of ovomucin in 1 per cent NaCl solution, the ovomucin concentration calculated on the basis of nitrogen determination, was diluted 1 : 4 with 0.85 per cent NaCl solution, well mixed and the respective virus added to give a final concentration of 40 to 60 agglutinating doses per ml. The mixture and an appropriate control were held in a water-bath at 37° C. Samples were withdrawn at suitable intervals for determination of the residual inhibitory titre.

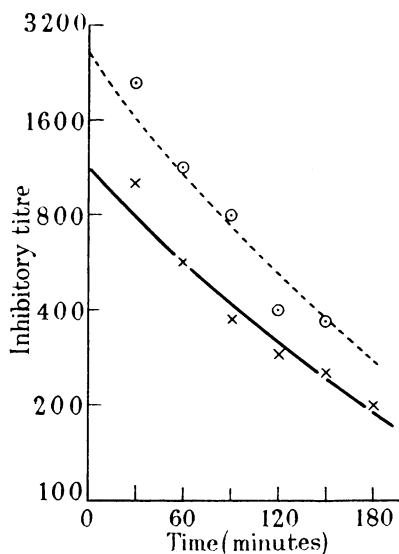


FIG. 1.

FIG. 1.—Action of active influenza virus on the virus inhibitor of ovomucin.

× ——— × Type A virus (Melbourne).

○ - - - ○ Type B virus (Lee).

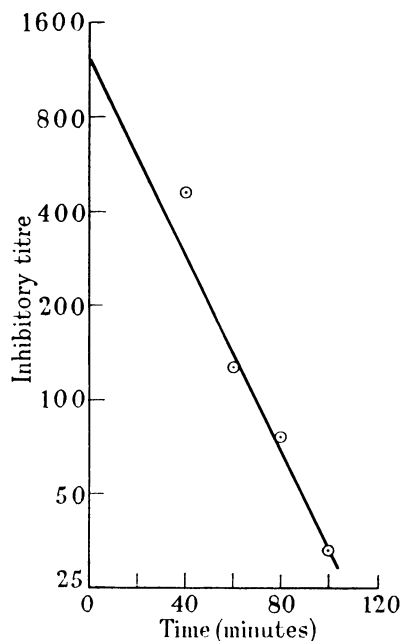


FIG. 2.

FIG. 2.—Action of the receptor destroying enzyme (RDE) on the virus inhibitor of ovomucin.

The results of two experiments, one carried out with influenza type A (Melbourne), the other with type B (Lee), are shown in Fig. 1. As may be seen from the slope of the lines, the inhibitory power of ovomucin is rapidly reduced by small quantities of active virus.

Reduction of the inhibitory power of ovomucin by RDE.

A corresponding technique, substituting RDE for virus and adding CaCl_2 in $\text{m}/200$ concentration, was used to investigate the effect of RDE on the inhibitory power of ovomucin. The final concentration of RDE in the reaction mixture was 80 units per ml. In order to determine the residual inhibitory titre of ovomucin, RDE was inactivated by preparing the serial dilutions in 0.85 per cent NaCl solution containing 0.005 M sodium hexametaphosphate as calcium de-ionizing agent. Fig. 2 illustrates the marked decrease in inhibitory titre of ovomucin by the action of RDE.

Reduction of the inhibitory power of ovomucin by trypsin.

The virus haemagglutinin inhibitor of the ovomucin fraction is very sensitive to trypsin action (Fig. 3). The loss by tryptic digestion of the capacity of ovomucin to inhibit haemagglutination by heated Lee virus is in agreement with the finding that the inhibitory power of normal serum is also rapidly destroyed by trypsin treatment (Burnet, McCrea and Anderson, 1947; Hirst, 1948; McCrea, 1948).

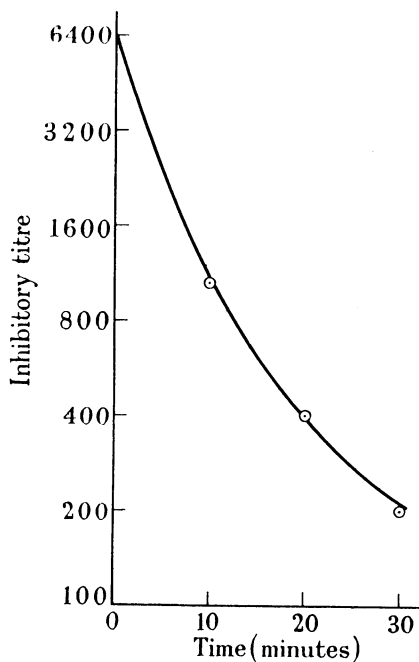


FIG. 3.

FIG. 3.—Action of trypsin on the virus inhibitor of ovomucin.

Conditions: To 1.0 ml. of 1 per cent ovomucin dispersion (in 0.85 per cent NaCl) added 1.0 ml. of 0.1 M phosphate-saline buffer (pH = 7.6), containing 4 mg. crystalline trypsin. Temperature 37° C. Samples for determination of residual inhibitory titre withdrawn at 10 min. intervals.

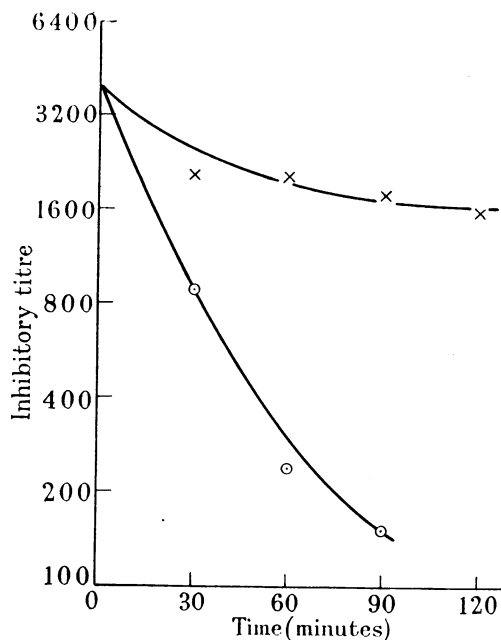


FIG. 4.

FIG. 4.—Reduction by active Lee virus of the inhibitory titre of ovomucin with NaCl concentration as the variable.

- — ○ 0.05 ml. active Lee virus added to 1.95 ml. of 0.4 per cent ovomucin dispersion in saline; 1.0 per cent final NaCl concentration.
 × — × As above, but 5.0 per cent final NaCl concentration. Inactivation of virus at 65° C. carried out in 5.0 per cent NaCl.

Effect of NaCl on the reduction by active virus of the inhibitory power of ovomucin.

Since the ovomucin is more easily and more uniformly dispersed in 5 per cent NaCl than in 1 per cent, the effect of NaCl on the virus-ovomucin interaction was investigated. It is evident from Fig. 4 that NaCl in 5 per cent concentration markedly inhibits the reduction by active Lee virus of the inhibitory power of ovomucin.

SUMMARY.

Of the various components of hen egg-white, only ovomucin has the power to inhibit haemagglutination by heated type B (Lee) influenza virus.

Ovomucin, as usually prepared, is a protein fraction rather than a single protein. It is always contaminated with lysozyme, in concentrations up to 15 per cent, part of which can be made to crystallize out directly from the ovomucin dispersion.

The inhibitory power of the ovomucin fraction is readily reduced by active influenza virus types A and B, by the receptor destroying enzyme of *Vibrio cholerae* and also by trypsin.

Sodium chloride in 5 per cent concentration inhibits markedly the reduction by active influenza virus of the inhibitory power of the ovomucin fraction.

We are greatly indebted to our colleague, Mr. E. L. French, for his most valuable co-operation in determining by biological assay the lysozyme content of the various ovomucin preparations.

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